Signal Transduction: A Novel p53 Phosphorylation Site within the MDM2 Ubiquitination Signal: I. PHOSPHORYLATION AT SER269 IN VIVO IS LINKED TO INACTIVATION OF p53 FUNCTION

Jennifer A. Fraser, Borivoj Vojtesek and Ted R. Hupp
doi: 10.1074/jbc.M110.143099 originally published online September 17, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.143099

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/09/17/M110.143099.DC1.html

This article cites 65 references, 24 of which can be accessed free at http://www.jbc.org/content/285/48/37762.full.html#ref-list-1
A Novel p53 Phosphorylation Site within the MDM2 Ubiquitination Signal

I. PHOSPHORYLATION AT SER^{269} IN VIVO IS LINKED TO INACTIVATION OF p53 FUNCTION

Jennifer A. Fraser^, Borivoj Vojtesek^1, and Ted R. Hupp^2

From the ^Institute of Genetics and Molecular Medicine, CRUK Cancer Research Centre, University of Edinburgh, Edinburgh EH4 2XR, Scotland, United Kingdom and the ^Masaryk Memorial Cancer Institute, Brno 656 53, Czech Republic

p53 is a thermodynamically unstable protein containing a conformationally flexible multiprotein docking site within the DNA-binding domain. A combinatorial peptide chip used to identify the novel kinase consensus site R(K/D) led to the discovery of a homologous phosphorylation site in the S10 β-strand of p53 at Ser^{269}. Overlapping peptide libraries confirmed that Ser^{269} was a phosphoacceptor site in vitro, and immunochemical approaches evaluated whether p53 is phosphorylated in vivo at Ser^{269}. Mutation or phosphorylation of p53 at Ser^{269} attenuates binding of the p53-specific monoclonal antibody DO-12, identifying an assay for measuring Ser^{269} phosphorylation of p53 in vivo. The mAb DO-12 epitope of p53 is masked via phosphorylation in a range of human tumor cells with WT p53 status, as defined by increased mAb DO-12 binding to endogenous p53 after phosphatase treatment. Phospho-Ser^{269}-specific monoclonal antibodies were generated and used to demonstrate that p53 phosphorylation is induced at Ser^{269} after irradiation with kinetics similar to those of p53 protein induction. Phosphomimetic mutation at Ser^{269} inactivated the transcription activation function and clonogenic suppressor activity of p53. These data suggest that the dynamic equilibrium between native and unfolded states of WT p53 can be modulated by phosphorylation of the conformationally flexible multiprotein binding site in the p53 DNA-binding domain.

Exposure to a wide variety of genotoxic and metabolic stresses leads to the activation of the p53 tumor suppressor protein, a sequence-specific DNA-binding protein and stress-activated transcription factor that controls and coordinates the expression of a battery of genes involved in transient growth arrest, DNA and cellular repair, and/or apoptosis (1). The function of the p53 protein is regulated post-translationally by enzymes that catalyze p53 ubiquitination, acetylation, and phosphorylation. In the absence of stress, the specific activity of p53 is suppressed by the action of E3 ubiquitin ligases like MDM2 that promote proteasomal degradation of the protein (2). In response to stress signals like DNA damage, this degradation program is suppressed, and sets of protein kinase pathways, notably ATM, trigger activation of the protein.

The mechanisms of p53 activation by phosphorylation at the most evolutionarily conserved phosphoacceptor sites has been assigned both biochemically and genetically. The phosphoacceptor sites in the p53 transactivation domain are the most highly conserved between vertebrates and invertebrates: phosphorylation at Ser^{20} stabilizes the interaction with acetyltransferases like p300 and in turn stimulates DNA-dependent acetylation (3, 4); phosphorylation at Ser^{15} can stimulate CBP binding and p53 acetylation (5); and phosphorylation at Thr^{18} can both stabilize p300 binding and reduce MDM2 binding (4, 6). The stabilization of p300 relates to the conversion of intrinsically unstructured activation motifs in p53 to a more helical character with a higher affinity for p300 (7). Mice with mutations at the equivalent Ser^{20} residue develop spontaneous B-cell lymphomas (8), and Ser^{15} mutant transgenes develop spontaneous late onset lymphoma (9). The Ser^{392} phosphoacceptor site in the C-terminal domain of p53 is the second most highly conserved class of phosphoacceptor site but only within vertebrates. Increased phosphorylation of p53 at the Ser^{392} site occurs in vivo after UV and ionizing radiation (10, 11), and this stimulates the sequence-specific DNA-binding function of p53 (12). Phosphorylation of p53 at Ser^{392} enhances the stability of the p53 tetramerization domain (13), and phosphomimetic mutation at codon 392 results in enhanced thermostability of the p53 tetramer (14), providing biophysical evidence for conformational changes of this phosphorylation on p53. Genetic studies in mice have shown that mutation of the CK2 site results in enhanced skin or bladder cancer in response to UV damage or carcinogen exposure (15, 16), and mouse embryo fibroblasts from such transgenic mice also have an attenuated p53 transcriptome (17). Further, the enhanced phosphorylation of p53 in the basal/stem cells of UV-irradiated human skin (11) is attributable to the transcriptional activation of ATM by ΔNp63 (18). Although these biochemical and genetic studies provide a paradigm for how phosphorylation can regulate p53 protein function at the most highly conserved phosphorylation sites, the effects of many other evolvent modifications on p53, including over 12 other phosphorylation sites and methylation sites, are only just now being defined at the biochemical and genetic level.

There is growing evidence that protein-protein interactions, although driven by globular domains, are regulated by intrinsically disordered motifs or linear peptide docking motifs (19).
These linear motifs might acquire structure upon binding to target protein or may themselves induce a specific structure by stabilizing the target protein in a specific conformation. p53 protein is a case in point; it is a thermodynamically unstable protein that has a large set of peptide-docking sites within its structural or unstructured domains that drive key protein-protein interactions that regulate its function, including ubiquitination and phosphorylation (20). The E3 ubiquitin ligase MDM2 is a prime example of this because at least two distinct linear peptide domain interaction sites are required for MDM2 to catalyze p53 ubiquitination. The primary binding site of MDM2 for p53 occurs at a peptide motif (FXXXWXXXL) in the intrinsically unstructured N-terminal domain of p53 through an interaction with the N-terminal hydrophobic pocket of MDM2 (21). The second MDM2 interaction site occurs through an interaction between the MDM2 acidic domain and a motif (SXXLXXGXXXF) in the conformationally flexible DNA-binding domain in p53 within the S9-S10 loop/S10β-sheet (22). This latter site forms the ubiquitination signal for E2-E3 (MDM2)-catalyzed ubiquitin transfer to the p53 tetramer (23). This site is notable in that it is also a site of pronounced conformational flexibility that reveals and “opening” or destabilization of the p53 DNA-binding domain (24). This conformationally flexible site is also notable in that it forms a docking site for the distinct class of protein kinases that phosphorylate p53 in its transactivation domain (25, 26), indicating that this region forms a multiprotein interaction site.

In order to determine whether p53 is directly phosphorylated at this multiprotein docking site, thus providing another layer of post-translational regulation, we took advantage of a tool involving kinase substrate profiling to define the linear interaction motifs that direct enzyme specificity and substrate utilization. Here we used chip peptide array technology to expand substrate utilization of the calmodulin kinase family members to define potential physiologically phosphoacceptor consensus sites using peptides from naturally occurring proteins. Indeed, PepChip technology has been used to successfully characterize the complex changes that occur within the epithelial esophageal kinome during the early transitional stages of carcinogenesis (27) and map the cellular phosphoproteome (28). After delineation of a novel consensus phosphoacceptor site coupled to homology searches for similar motifs in the p53 DNA-binding domain, we identified a novel phosphorylation site in the S10β-sheet region of p53 at Ser269. This region is notable in being (i) a site of conformational flexibility in mutant gain-of-function p53 (24); (ii) the ubiquitin signal for MDM2-mediated ubiquitination of p53 (23); and (iii) a docking site for a range of protein kinases that phosphorylate the transactivation domain of p53 (25). A range of approaches were used to demonstrate that Ser269 phosphorylation can occur on endogenous WT p53 in cells and that phosphomimetic mutation at codon 269 results in the production of inactive WT p53. An accompanying paper describes the biophysical basis for p53 inactivation by phosphorylation of p53 at Ser269 and involves primarily increases in the thermoinstability of the core DNA-binding domain of p53. These data highlight the existence of a novel kinase pathway that can regulate the dynamic range of conformations in WT p53 and that can produce a mutant-like conformation on WT p53.

**Materials and Methods**

**Reagents and Plasmids**—N-terminally tagged biotinylated peptides with an SGSG spacer were obtained from Mimotopes (Carlton, Australia). Anti-p53 antibodies were DO-1, DO-12, PAAb240, PAAb1620, CM-1, Ab-1 (anti-p21, Cell Signaling), and 2A10 (anti-Mdm2). pcDNA 3.1 p53 and pCMV-Mdm2 plasmids were described (23). The p53 gene was cloned into the pExpr vector (IBA Systems), generating a construct with an N-terminal streptavidin tag. Single amino acid mutations were introduced into wild type p53 at Ser269 according to the QuikChange site-directed mutagenesis kit (Stratagene) using pcDNA 3.1 p53, pExpr p53, and pRSET p53 DNA core domain as templates, and the following oligonucleotides were used for mutagenesis (underlined): p53 S269A, 5′-gga cgg aac gtt ctt gag tgt cc-3′ (forward primer) and 5′-cg cac ctc aaa agc gtt cgg tcc c-3′ (reverse primer); p53 S269D, 5′-ctg gga cgg aac agc gtt gag tgt cc-3′ (forward primer) and 5′-cg cac ctc aaa agc gtt cgg tcc cag-3′ (reverse primer).

**Kinase Assays**—PepChip kinase slides were obtained from Mimotopes Pty. Ltd. Protein kinases were mixed with 10 μM ATP and 300 μCi/ml [γ-33P]ATP (3000 Ci/mmol) in 50 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 0.8 mM EDTA, 5% glycerol, and 0.01% Brij-35, in the presence or absence of 75 μM Box V peptide (30). The reaction mixtures were spotted onto the PepChip slide and covered before they were transferred into a humidified chamber and incubated at 30 °C for 3 h. The PepChip slides were washed once with phosphate-buffered saline containing 1% Triton X-100 and twice with 2 M NaCl containing 1% Triton X-100. [γ-33P]ATP incorporation was visualized using a PhosphorImager (Storm 840, Amersham Biosciences). Peptide sequence information pertaining to the individual spots was obtained by overlaying the image with the reference grid provided by Pepscan Systems B.V. (Lelystad, Netherlands). Peptide kinase reactions were carried out as described previously (30), using 150 ng of purified recombinant protein kinase and 1 μg of peptide substrate.

**Peptide ELISA**—Biotinylated unphosphorylated and phosphorylated peptides were captured onto ELISA wells coated with streptavidin and blocked with 3% BSA in PBS-Tween as described previously (31).

**Cell Culture, Transfection, and Analysis**—All tissue culture medium was obtained from Invitrogen and was supplemented with 10% fetal bovine serum. H1299 cells were cultured in RPMI 1640 medium; A375, HEK-293, and MCF-7 cells were cultured in DMEM, and HCT-116 p21 null cells were grown in McCoy’s medium. Cells were harvested and lysed using urea lysis buffer as described previously (30) unless otherwise stated. For luciferase assays, H1299 cells were transfected the following day with 30 ng of pCMV Renilla and 70 ng of either p21-luc or Bax-luc and plasmids encoding p53 wild type, p53S269A, or p53S269D. Cells were lysed with lysis buffer according to the dual luciferase assay kit (Promega), and luciferase activity was quantified using a luminometer (Fluoroskan Ascent FL). For cellular fractionation, cells were treated with 10 J cm−2 UVC and grown for a further 6 h at 37 °C prior to fractionation using the S-PEK.
Phosphorylation of p53 in the Ubiquitination Signal

subcellular fractionation kit (Calbiochem). For immunoprecipitation, cell lysates (100 ng) were precleared with protein G beads (Sigma) for 1 h before incubation with 1 μg of DO-1, PAAb1620, or PAb240 at 4 °C. For immunoprecipitation of endogenous phospho-Ser\textsuperscript{269} p53, mouse monoclonal antibodies were preadsorbed to protein G beads overnight before incubation with precleared lysates and washed and eluted as above. For clonogenic survival assays, H1299 cells were transfected with pcDNA p53 constructs, and colonies were selected using Geneticin as described previously (32).

**Phosphatase Treatment of Nitrocellulose Membranes**—Nitrocellulose membranes were incubated in 50 mM Tris, pH 7.5, 5 mM DTT, 0.1 mM EDTA, 2 mM MnCl\textsubscript{2}, and 80 units of λ-protein phosphatase (Sigma) for 1 h at 30 °C (33). The degree of epitope unmasking was determined by quantifying the band intensity using Scion Image software.

**RESULTS**

*In Vitro Kinase Screens Identify a Novel Phosphoacceptor Site at Ser\textsuperscript{269} in the DNA-binding Domain of p53*—Because protein-protein interactions are driven in part by intrinsically disordered linear peptide motifs, we previously screened a peptide library with MDM2 protein to acquire novel MDM2 consensus peptide binding motifs. These peptide motifs were scanned for homology to sites in the tumor suppressor protein p53, thus identifying a second MDM2 binding site in the p53 DNA-binding domain (22, 35). Similarly, in this report, we screened selected kinase superfamily members using a kinase-peptide array that contains 192 naturally occurring phosphoacceptor sites to define a broader consensus site for protein kinases. These consensus sites in turn could be scanned for homology to motifs in p53 that might reveal novel p53 phosphorylation sites.

When kinases are screened in this assay, a range of peptide substrates were identified (Fig. 1A shows a representative screen using DAPK-1 (i.e. DAPK core kinase domain)). DAPK targeted 68 of the 192 peptides on the chip (Fig. 1A), and these peptides covered a wide range of cellular proteins (supplemental Table I). Although the library of peptides on the chip does not comprehensively represent the diversity of the human proteome and known substrates of DAPK, such as p21, p53, and myosin light chain, are not represented, there were several peptide hits that were in keeping with the function of DAPK in regulating the cytoskeleton, such as vimetin. The majority of peptides targeted by DAPK core contained a serine residue as the phosphoacceptor (52 of 68 peptides), whereas only a small proportion contained a threonine as the phosphoacceptor (5 of 68 phosphorylated peptides). Several peptides contained more than one potential phosphoacceptor site (e.g. peptide 1, 4, or 8 (LRRSSSVGY, PGGSTPVSS, or KTTASSFRK)), and in such cases, the central residue was taken as the phosphoacceptor. Analysis of the relative abundance of the various amino acid residues within the peptides targeted by DAPK (Table I) showed a strong selection for peptides with leucine and lysine residues at the −1-position and a strong preference for basic residues, such as lysine and arginine, upstream of the phosphoacceptor site (30, 36, 37). The most prominent residues at the −1-position were more non-polar/hydrophobic residues, such as proline, glycine, and alanine. Downstream of the phosphoacceptor serine, there was a high prevalence of basic residues, such as arginine and lysine, as well as proline residues at the +1- and +2-positions; however, there was also an increased abundance of valine and phenylalanine residues. This is also in keeping with previous findings that showed that DAPK has a strong preference for hydrophobic leucine, valine, and phenylalanine residues downstream of the phosphorylated serine (36, 37). There was also a positive preference for alanine residues at the +3-position and acidic residues, such as glutamic and aspartic acid, at +2 and +3. The relative abundance of the various amino acids was tallied to generate a consensus sequence, and the simplest derived consensus for DAPK substrates was determined to be (K/L)(R/K)(P/G/A)(S/P/V/F)(R/K/D)(A/E/P)(K/S/P) (Table 1). The minimal consensus site of RXSF(D/K) obtained (Fig. 1B) was then screened for homology to p53 and identified a site in the p53 tumor suppressor protein as lysine and arginine, upstream of the phosphoacceptor site (30, 36, 37). The most prominent residues at the −1-position were more non-polar/hydrophobic residues, such as proline, glycine, and alanine. Downstream of the phosphoacceptor serine, there was a high prevalence of basic residues, such as arginine and lysine, as well as proline residues at the +1- and +2-positions; however, there was also an increased abundance of valine and phenylalanine residues. This is also in keeping with previous findings that showed that DAPK has a strong preference for hydrophobic leucine, valine, and phenylalanine residues downstream of the phosphorylated serine (36, 37). There was also a positive preference for alanine residues at the +3-position and acidic residues, such as glutamic and aspartic acid, at +2 and +3. The relative abundance of the various amino acids was tallied to generate a consensus sequence, and the simplest derived consensus for DAPK substrates was determined to be (K/L)(R/K)(P/G/A)(S/P/V/F)(R/K/D)(A/E/P)(K/S/P) (Table 1). The minimal consensus site of RXSF(D/K) obtained (Fig. 1B) was then screened for homology to p53 and identified a site in the p53 tumor suppressor protein as lysine and arginine, upstream of the phosphoacceptor site (30, 36, 37). The most prominent residues at the −1-position were more non-polar/hydrophobic residues, such as proline, glycine, and alanine. Downstream of the phosphoacceptor serine, there was a high prevalence of basic residues, such as arginine and lysine, as well as proline residues at the +1- and +2-positions; however, there was also an increased abundance of valine and phenylalanine residues. This is also in keeping with previous findings that showed that DAPK has a strong preference for hydrophobic leucine, valine, and phenylalanine residues downstream of the phosphorylated serine (36, 37). There was also a positive preference for alanine residues at the +3-position and acidic residues, such as glutamic and aspartic acid, at +2 and +3. The relative abundance of the various amino acids was tallied to generate a consensus sequence, and the simplest derived consensus for DAPK substrates was determined to be (K/L)(R/K)(P/G/A)(S/P/V/F)(R/K/D)(A/E/P)(K/S/P) (Table 1). The minimal consensus site of RXSF(D/K) obtained (Fig. 1B) was then screened for homology to p53 and identified a site in the p53 tumor suppressor protein as lysine and arginine, upstream of the phosphoacceptor site (30, 36, 37). The most prominent residues at the −1-position were more non-polar/hydrophobic residues, such as proline, glycine, and alanine. Downstream of the phosphoacceptor serine, there was a high prevalence of basic residues, such as arginine and lysine, as well as proline residues at the +1- and +2-positions; however, there was also an increased abundance of valine and phenylalanine residues. This is also in keeping with previous findings that showed that DAPK has a strong preference for hydrophobic leucine, valine, and phenylalanine residues downstream of the phosphorylated serine (36, 37). There was also a positive preference for alanine residues at the +3-position and acidic residues, such as glutamic and aspartic acid, at +2 and +3. The relative abundance of the various amino acids was tallied to generate a consensus sequence, and the simplest derived consensus for DAPK substrates was determined to be (K/L)(R/K)(P/G/A)(S/P/V/F)(R/K/D)(A/E/P)(K/S/P) (Table 1). The minimal consensus site of RXSF(D/K) obtained (Fig. 1B) was then screened for homology to p53 and identified a site in the p53 tumor suppressor protein as lysine and arginine, upstream of the phosphoacceptor site (30, 36, 37). The most prominent residues at the −1-position were more non-polar/hydrophobic residues, such as proline, glycine, and alanine. Downstream of the phosphoacceptor serine, there was a high prevalence of basic residues, such as arginine and lysine, as well as proline residues at the +1- and +2-positions; however, there was also an increased abundance of valine and phenylalanine residues. This is also in keeping with previous findings that showed that DAPK has a strong preference for hydrophobic leucine, valine, and phenylalanine residues downstream of the phosphorylated serine (36, 37). There was also a positive preference for alanine residues at the +3-position and acidic residues, such as glutamic and aspartic acid, at +2 and +3. The relative abundance of the various amino acids was tallied to generate a consensus sequence, and the simplest derived consensus for DAPK substrates was determined to be (K/L)(R/K)(P/G/A)(S/P/V/F)(R/K/D)(A/E/P)(K/S/P) (Table 1). The minimal consensus site of RXSF(D/K) obtained (Fig. 1B) was then screened for homology to p53 and identified a site in the p53 tumor suppressor protein.
Phosphorylation of p53 in the Ubiquitination Signal

The derived consensus sequence is as follows: (K/L)(R/K)R(P/G/A)(E/D)(V/F/V)(R/K/D)(A/E/P)(K/S/P).

<table>
<thead>
<tr>
<th>Position</th>
<th>Ser</th>
<th>Thr</th>
<th>Tyr</th>
<th>Lys</th>
<th>Leu</th>
<th>Arg</th>
<th>Pro</th>
<th>Gly</th>
<th>Ala</th>
<th>Val</th>
<th>Ile</th>
<th>Asp</th>
<th>Glu</th>
<th>Asn</th>
<th>Phe</th>
<th>Gln</th>
<th>Trp</th>
<th>Met</th>
<th>His</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoacceptor site</td>
<td>52</td>
<td>9</td>
<td>7</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Quantifying the abundance and positioning of the amino acid residues within the DAPK peptide targets (data derived from supplemental Table I)

This region of p53 containing Ser<sup>269</sup> or Ser<sup>260</sup>/Ser<sup>261</sup> phosphoacceptor sites (Fig. 3A) were used in kinase assays to determine whether these serine residues are bona fide phosphoacceptor sites. Only peptides containing the Ser<sup>269</sup> site can function as substrates for DAPK (Fig. 3B, lanes 5–9 versus lanes 1–4), consistent with the kinase-peptide scan in Fig. 1, where a bulky hydrophobic residue flanking the phosphoacceptor site plays a role in phosphorylation. By contrast, another kinase we evaluated (Chk1) is a relatively poor kinase toward peptides containing the Ser<sup>269</sup> phosphoacceptor site, whereas it demonstrates better specificity for the peptide with double phosphoacceptor sites at Ser<sup>260</sup>/Ser<sup>261</sup> (Fig. 3C, lanes 2–5 versus lanes 6–9). Together, these data indicate that the flexible linker of p53 has a phosphoacceptor site at Ser<sup>269</sup> in vitro that shares homology with members of the calcium-calmodulin kinase superfamily that can target the RXSF(D/E) motif.

In Vivo Evidence for p53 Phosphorylation at Ser<sup>269</sup> Defined by DO-12 mAb Epitope Masking—We next evaluated whether in vivo evidence could be acquired for Ser<sup>269</sup> phosphorylation. If so, this would make biochemical and cellular analysis of Ser<sup>269</sup> modification more physiologically relevant. Two approaches were taken to examine Ser<sup>269</sup> phosphorylation in cells: (i) epitope masking using a monoclonal antibody whose binding site overlaps with Ser<sup>269</sup> and (ii) phosphospecific antibody generation to phospho-Ser<sup>269</sup>. A notable feature of the conformationally flexible motif containing Ser<sup>269</sup> is that it overlaps with the DO-12 monoclonal antibody epitope (Fig. 2) (24); this epitope (amino acids 255–270) is normally constrained in wild type p53 and in mutant p53 due to conformational changes that expose the epitope. This change in epitope exposure due to p53 mutations is thought to be due to the changes in the dynamic range in p53 conformational states, according to the ensemble model of allostery.

The Ser<sup>269</sup> residue is located at the end of the DO-12 epitope (amino acids 255–270) (Fig. 2). Key phosphorylation sites on p53 are often found within the epitopes of several monoclonal antibodies (such as DO-1 (calcium-calmodulin kinase superfamily superfamily that shares homology with members of the calcium-calmodulin kinase superfamily that can target the RXSF(D/E) motif).
family site at Ser\textsuperscript{269} (31), PAB421 (PKC site at Ser\textsuperscript{371/376} (42), ICA-9 (CK2 site, Ser\textsuperscript{392}), or MDM2 (2A10, ATM site at Ser\textsuperscript{399})) (43), and monoclonal antibody binding to these epitopes is masked by phosphorylation. As such we evaluated whether mutation or substitution of p53 at Ser\textsuperscript{269} attenuated DO-12 monoclonal antibody binding. If so, this would provide an indirect assay for determining whether phosphorylation occurs at Ser\textsuperscript{269} on endogenous p53 \textit{in vivo}. DO-12 mAb binding to peptides derived from this region of p53 confirmed that Ser\textsuperscript{269} and Phe\textsuperscript{270} comprise a portion of the DO-12 epitope (Fig. 4A), raising the possibility that Ser\textsuperscript{269} phosphorylation could affect mAb-DO-12 binding. Phosphopeptides were generated within the DO-12 epitope that contain phosphate at Ser\textsuperscript{269}, Ser\textsuperscript{261}, or Ser\textsuperscript{260}. When DO-12 binding to these substituted peptides was measured, only Ser\textsuperscript{269} phosphorylation attenuated the binding of the monoclonal antibody (Figs. 4B and 5). By contrast, Ser\textsuperscript{261} or Ser\textsuperscript{260} phosphopeptides actually exhibited elevated binding stability toward DO-12 (Fig. 4B). Mutagenesis of codon 269 in full-length p53 to alanine or the phosphomimetic aspartate resulted in masking of the DO-12 epitope (Fig. 5, A and B), indicating that the integrity of Ser\textsuperscript{269} is required for DO-12 to bind stably. As a control, binding of another conformationally sensitive p53 monoclonal antibody, PAb240 (epitope 209–214) (44), was examined, and PAb240 binding was not inhibited by mutation of codon 269 (Fig. 5C), indicating that mutation and phosphorylation at Ser\textsuperscript{269} specifically masks the DO-12 epitope.

A range of cell lines containing wild type p53 protein (including malignant melanoma (A375), mammary adenocarcinoma (MCF-7), colorectal carcinoma (HCT-116), and human embryonic kidney cells (HEK-293)) were lysed in denaturing urea buffer to preserve endogenous p53 phosphorylation sites, and total p53 protein levels were measured (Fig. 6A, lanes 1–4). Immuno-
Phosphorylation of p53 in the Ubiquitination Signal

In vivo phosphorylation of endogenous p53 at serine 269; detection by phosphatase unmasking of antibody epitopes. A–C, phosphatase activity exposes the DO-12 epitope on endogenous p53. Lysates from A375, MCF-7, HEK, and HCT-P1-2 cells were resolved by electrophoresis, and immunoblots were probed with antibody for total p53 (A; total p53), DO-12 (B; epitope 255–270), or PAb240 (C; epitope 209–214) by immunoblotting.

D, The total p53 protein was distributed into all three compartments shown (Fig. 7B, lanes 1–3) with little evidence of DO-12-reactive p53 protein (Fig. 7C, lanes 1–3). The total p53 protein was distributed into all three compartments shown (Fig. 7B, lanes 1–3) with little evidence of DO-12-reactive p53 protein (Fig. 7C, lanes 1–3). This is consistent with the data acquired using denaturing urea lysis (Fig. 6) and suggests that the buffers used do not permit dephosphorylation of the DO-12 epitope. DO-12-reactive p53 protein could not be detected after phosphatase treatment of blots presumably because of the large dilutions used in the chemical fractionations (Fig. 7A) relative to whole cell denaturing lysis buffer (Fig. 6). As such, we also evaluated whether DNA damage changes DO-12 epitope masking of p53 and in which compartment this occurred. In response to irradiation at doses that activate p53 (data not shown), there is an increase in p53 protein in the nuclear fraction (Fig. 7B, lane 6 versus lanes 1–5), consistent with previous reports that endogenous p53 protein is largely stored in cytosolic fractions and is transported into the nucleus after DNA damage by a dynein-dependent pathway (46). Under conditions where p53 protein is stabilized by irradiation, we could detect DO-12-reactive p53 protein in the nucleus (Fig. 7C, lane 6 versus lanes 1–5), and this was increased by phosphatase treatment.
of the immunoblots (Fig. 7D, lane 6 versus lanes 1–5). The loading controls for the subcellular fractionation are highlighted in Fig. 7, E–H. These data suggest that p53 can be phosphorylated in the basal state in the DO-12 epitope but that DNA damage induces a mixed pool of DO-12-reactive (i.e. non-phosphorylated) and DO-12-non-reactive p53 protein (i.e. phosphorylated).

**In Vivo Evidence for p53 Phosphorylation at Ser269** Defined by the Use of Novel Phosphospecific Ser269 mAbs—Although phosphatase-sensitive epitope masking of p53 has been a standard method of defining phosphorylation of p53 at some sites, phosphospecific antibodies are the more generally useful tool now used to measure steady-state levels of a post-translational modification. We have previously generated phosphospecific monoclonal antibodies as tools for investigating p53 phosphorylation in vivo (47), including Ser392 and Ser315 (48, 49). A panel of phosphospecific monoclonal antibodies was generated to phospho-Ser269-containing peptides that demonstrated specificity for the peptide from the MDM2 ubiquitination signal (LGRNPSEVR, where pS represents phosphoserine) (Fig. 8A). An alanine scan phosphopeptide screen indicated that the phospho-Ser269-specific monoclonal antibodies displayed a requirement for key amino acids surrounding the phosphoserine residue (Fig. 8A, i–iii). We used these monoclonal antibodies to determine whether similar evidence could be acquired for radiation-induced Ser269 phosphorylation of p53 protein that correlated with DO-12 epitope masking.

When cells were exposed to UV or x-ray radiation, the standard induction of p53 protein could be confirmed (Fig. 8B). Immunoprecipitation of p53 with a monoclonal antibody and direct blotting with a polyclonal antibody could confirm that the immunoprecipitation assay could be used to measure increases in p53 protein levels (Fig. 8C). When the monoclonal antibody 2.1 was used under the same conditions, an increase in Ser269-phosphorylated p53 protein could be detected whether using x-ray or UV irradiation (Fig. 8D). A time course measuring increases in p53 protein levels (Fig. 8E) or phospho-Ser269 p53 protein levels (Fig. 8F) indicated that the kinetics of induction of p53 phosphorylation at Ser269 was similar to that of total p53 protein. Thus, using either a mAb epitope masking assay or direct phospho-Ser269-specific mAb binding assay, we present evidence that phosphorylation at Ser269 can occur in vivo and that it increases in response to DNA damage.

**An Inactivating Function for Ser269 Phosphorylation of p53**—In order to propose a function for Ser269 phosphorylation of p53, mutagenesis was performed to produce a phosphomimetic p53 mutant. However, one of the difficulties with studying p53 protein–protein interactions in the core DNA-binding domain by mutation is that changes in the majority of codons “inactivate” p53, making such an analyses difficult to interpret. Examination of codon 269 mutations in human cancers shows the predominant changes, including Serine mutation to Thr, Ile, Gly, Leu, Asn, Arg, and Cys (see the International Agency for Research on Cancer Web site). Because these mutations are presumably inactivating, we examined whether we could first identify “neutral” mutations at codon 269 that do not inactivate p53. The p53 mutant p53S269A did not lose activity; indeed, the specific activity was elevated relative to wild type p53 as defined by MDM2 protein induction (Fig. 9, lane 4 versus lane 3). The enhanced function of p53S269A was not observed using transient transfection of bax or p21Luc reporters; nevertheless, the S269A mutant was as active as WT p53 (Fig. 9, C and D), indicating that a mutation does not a priori “inactivate” p53 protein function.

As such, we also examined whether a phosphomimetic mutation at codon 269 would likewise stimulate, inactivate, or demonstrate no change in p53 activity. The mutant p53S269D was unable to induce MDM2 or p21 proteins (Fig. 9, A and B), suggesting that adding a negative charge at codon 269 can inacti-
These data suggest that Ser269 phosphorylation will most likely produce a functionally mutant wild type p53 protein and therefore represent an inactivating phosphorylation. The transcriptional activities of p53S269D mutant toward luciferase cassettes containing \( p21 \) and \( Bax \) promoter elements were also tested. Transfection of the phosphomimetic p53S269D mutant was unable to induce transcription from \( p21 \) and \( Bax \) promoter elements (Fig. 9, C and D). A clonogenic assay was also used to evaluate whether the phosphomimetic mutation altered WT p53 function. Compared with the wild type or p53S269A, which exhibit growth-suppressing activities (Fig. 10, B and C versus A), the S269D mutation inactivated the growth-suppressive function of p53 (Fig. 10, D versus B). These cellular findings (Figs. 9 and 10) indicate that phosphomimetic mutation of p53 at Ser269 inhibits WT p53 function and together suggest that phosphorylation of p53 \( \textit{in vivo} \) at serine 269 produces a pool of transcriptionally attenuated p53 protein.

**DISCUSSION**

There is growing evidence that a majority of protein-protein interactions in higher eukaryotes are regulated by intrinsically disordered motifs (50). p53 protein has large regions containing
intrinsically unstructured motifs, and the core DNA-binding domain is thermodynamically unstable. These regions regulate protein-protein interactions that control p53 activity. One recently identified conformationally flexible motif harbors both the MDM2 ubiquitination signal and protein kinase docking sites in the p53 DNA-binding domain (Fig. 11). This multifunctional protein interaction site thus has a potential to play a fundamental role in p53 activation and its inhibition. In order to determine whether p53 is phosphorylated within this multiprotein docking site in the MDM2 ubiquitination signal, we took advantage of a tool involving kinase substrate profiling to define the linear interaction motifs that direct enzyme specificity and substrate utilization. Here we used chip peptide array technology to expand substrate utilization of the calmodulin kinase family members, including DAPK and Chk2, to define potential physiological phosphoacceptor consensus sites using peptides from naturally occurring proteins. PepChip technology has been used to successfully characterize the complex changes that occur within the epithelial esophageal kinome during the early transitional stages of carcinogenesis (27) and map the cellular phosphoproteome (28). After delineation of a novel consensus phosphoacceptor site coupled to homology searches for similar motifs in the p53 DNA-binding domain, we identified a novel phosphorylation site in the S10 β-sheet region of p53 at Ser269. This region (Fig. 11) is notable in being (i) a site of conformational flexibility in mutant gain-of-function p53 (24); (ii) the ubiquitin signal for MDM2-mediated ubiquitination of p53 (23); and (iii) a docking site for a range of protein kinases that phosphorylate the transactivation domain of p53 (25). A range of approaches were used to demonstrate that Ser269 phosphorylation can occur on endogenous WT p53 in cells and that phosphomimetic mutation results in the production of inactive WT p53. An accompanying paper (51) describes the biophysical basis for p53 inactivation by phosphorylation of p53 at Ser269 and involves primarily increases in the thermostability of the core DNA-binding domain of p53. These data highlight the existence of a novel kinase pathway that can regulate the dynamic range of conformations in WT p53 and that can produce a mutant-like conformation on WT p53.

Most phosphorylation sites characterized to date play a role in p53 activation. These include (i) phosphorylation in the transactivation domain (the BOX-I domain) at Thr18 and Ser20, which stabilizes the activator p300 (3, 4) and/or destabilizes the inhibitory protein MDM2 (31, 52); (ii) phosphorylation at the C-terminal CK2 site, which stimulates specific DNA binding (42); (iii) PKC phosphorylation in the C-terminus that can create a 14-3-3 phosphopeptide binding motif and stimulate the specific DNA binding function of p53 (53); and (iv) CDK phosphorylation at Ser215 after DNA damage that can stimulate the specific DNA-binding function of p53 (49).
small growing number of phosphorylation events that are linked to p53 inactivation. These include (i) phosphorylation by the CK2 component of the COP9 signalosome that triggers p53 degradation (54); (ii) phosphorylation at Ser315 by a GSK3-dependent pathway that can stimulate p53 nuclear export (55); and (iii) phosphorylation by Aurora kinase at either Ser215 or Ser315, which was reported to inactivate p53 by an undefined mechanism (56, 57). Thus, despite the fact that there are a growing number of inactivating phosphorylation sites on p53, there are few mechanistic data on how this regulates protein-protein interactions that lead to p53 inactivation. In this report, in analyzing a novel phosphorylation site on p53 using aspar-tate, phosphomimetic mutants, our data indicate that Ser269 phosphorylation would be inactivating with respect to p53-dependent transcription and clonogenic suppression. We do not know as of yet the physiological kinase that targets the Ser269 site on p53 and whether DAPK or CHK1 could actually target Ser269 in vivo. Previous work on protein kinases has shown dual functions: (i) the CK1 enzyme can either activate p53 (via virus or TGF-β pathway) (26, 58) or suppress p53 functions (59, 60), depending upon the input signal, and (ii) the same phosphorylation site on p53 (Ser315) can be activating after DNA damage (CDK2) (49, 61) or inactivating in cycling cells (by GSK3 or by Aurora) (55), suggesting that context is important for kinase signaling. Thus, by this logic, if DAPK is a Ser269 kinase in vivo, then it would be consistent with its previously defined dual function; DAPK can be activating for p53 with an ARF-oncogene signal input (62), but it could be inactivating for p53 given an EGF input signal where DAPK inactivates TSC2, resulting in mTOR stimulation (63). However, we do not know that DAPK is the real in vivo kinase for Ser269. DAPK is part of the calcium-calmodulin superfamily, which can target the XSEF motif (25) (Table 1). Thus, we do not know that DAPK and CHK1 are, necessarily, the Ser269 kinases in cells; this is the work we are currently investigating to see whether the calcium-calmodulin superfamily or other families represent the in vivo kinase for the Ser269 site.

**REFERENCES**

Phosphorylation of p53 in the Ubiquitination Signal